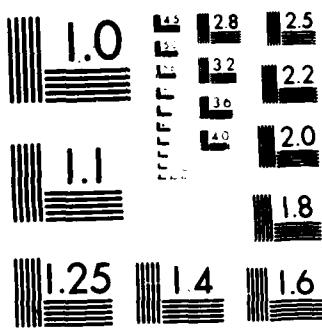


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D P NAGLE ET AL 27 MAR 87 87-00014-01 N00014-86-K-0222  
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Research on the genetics of methane producing archaeabacteria is directed at fundamental problems of mutant construction, gene transfer by transformation, and construction of vectors which facilitate gene transfer. From the thermophilic methanogen, Methanobacterium thermoautotrophicum, a set of mutants was obtained which included strains resistant to antibiotics or antimetabolites. Also, by using a new mutagenesis protocol, formic acid auxotrophs were obtained, whose physiology is of great interest. Genetic transfer via DNA-dependent natural transformation was achieved for two markers, 5-fluorouracil-resistance, and 6-mercaptopurine resistance. These markers were partially linked, although the genetic distance has not been determined. Vector construction is underway to link methanogen genes with an Escherichia coli plasmid. The recombinant DNA will be mutagenized by transposon insertion and then transformed back into M. thermoautotrophicum. The insertion can be used to locate the wild-type sequences from the methanogen by southern blotting of

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restriction fragments. The characterization of auxotrophic and resistant mutants, and the isolation of new mutants is ongoing, as is optimization of the transformation system. In the ten months since the start of the project, significant progress towards the long term goal of understanding genetic regulation in methanogens has been made.



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## Progress Report, N00014-K-0222

## Development of a Gene Cloning System in Methanogens.

## 1. SUMMARY OF PROJECT GOALS

- a) to obtain auxotrophic mutants, and antibiotic-resistant or antimetabolite-resistant mutants.
- b) to refine our methods for DNA-dependent transformation of Methanobacterium thermoautotrophicum and use this system in the construction of strains to study the physiology of this organism.
- c) to develop a shuttle vector based upon an M. thermoautotrophicum plasmid (see section 3b). Homologous chromosomal genes, drug resistance genes, and genes coding for enzymes that produce colored products will be tested as markers for plasmid transformation. A functional plasmid vector will allow us to study gene expression in an M. thermoautotrophicum background.
- d) the long term goal of this work is to understand the regulation of genes in methane-producing archaeabacteria.

## 2. RECENT ACCOMPLISHMENTS

Since the project started in June, 1986, we have made significant progress in a number of areas. In the DATA SYNOPSIS section we list several manuscripts which are in preparation, and we have additional publishable data in hand.

a) Our collection of mutants has been augmented by several spontaneously occurring resistant mutants, and doubly-resistant mutants derived from them. We tested an array of approximately 70 compounds on M. thermoautotrophicum and found several which are strong inhibitors. We purified a series of clones, which include resistances to mercaptapurine, azahypoxanthine, diazauracil, kanamycin, mitomycin C, and fluorouracil-mercaptapurine and fluorouracil-fluorodeoxyuridine. In addition, we have tested the use of hydroxylamine or ethylmethanesulfonate as mutagens in M. thermoautotrophicum. A protocol based upon hydroxylamine mutagenesis followed by bacitracin-enrichment of our kanamycin resistant strain yielded 24 auxotrophic clones. Ten of these have been characterized in a nutrient pool-elimination study. All 10 require a pool of volatile fatty acids, and in addition, two appear to require vitamins and a third requires an amino acid pool which contains twenty different amino acids. Thus, these three are likely to be triple mutants. We found that three of the 10 fatty acid-requiring strains are formate auxotrophs, a surprising and very interesting requirement.

b) We have explored methods for gentle lysis of M. thermoautotrophicum in order to obtain high molecular weight DNA. The most promising of these utilizes sub-bactericidal levels of bacitracin, followed by treatment with Proteinase K and detergent. This method works most of the time, but remains to be optimized. We used the bacitracin lysis technique to screen our laboratory strains for the presence

of cryptic plasmids. Our strain of M. thermoautotrophicum had maintained its plasmid during 5 years of subculture in the absence of selection for the plasmid. Previously it appeared that the plasmid was lost from this strain. A more recently obtained strain from the DSM culture collection, and the fluorouracil-resistant strain RTAE-1 derived from it contained the plasmid. We found no evidence for a plasmid in the fastidious methanogen Methanomicrobium mobile.

c) Culture of methanogens has been optimized in our laboratory. We have developed a simple mineral medium which can be made from dry chemicals, gas-exchanged, and placed in the autoclave within 18 minutes. In the 3-liter fermentor we obtained 4.2 g wet weight of M. thermoautotrophicum cells per liter in 60 hr in this medium (see section 3e).

d) The development of a DNA-dependent transformation system has been a major breakthrough. Table 1 contains some of our data which indicate that 5-fluorouracil and 6-mercaptopurine-resistance were transferred to the recipient wild-type cells. In fact, these markers appear to be linked. Spontaneous resistance appears at a frequency of  $10^{-8}$  for each of the markers. We have never observed spontaneous double resistance. The transformation system appears to be saturated at about 0.1 ug DNA/plate, and is more efficient for high molecular weight DNA, than for sheared DNA containing the same genetic markers.

Table 1. Results of transformation experiments with *Methanobacterium thermoautotrophicum* (Marburg) on plates.

Source of DNA	No. colonies tested	% colonies with transformants		
		FU <sup>R</sup>	MP <sup>R</sup>	FU <sup>R</sup> -MP <sup>R</sup>
<u>M. thermoautotrophicum</u>				
Wild Type	172	0.6	0	0
Strain RTAE-1 (FU <sup>R</sup> )	417	2.4	0	0
Strain RTVW-1 (FU <sup>R</sup> -MP <sup>R</sup> )	223	3.1	4.5	3.1
<u>Halobacterium volcanii</u>				
	99	0	0	0
<u>Escherichia coli</u>				
	216	0	0	0
None	348	0	0	0

abbreviations: FU, 5-fluorouracil; MP, 6-mercaptopurine.

Colonies were grown on plates in the presence of DNA under non-selective conditions, screened for the presence of resistant cells by replica plating, and these were rechecked for resistance.

e) Facilities. In February we installed brand new large-scale fermentors (10 and 100 liter, computer-controlled systems), are adding to our thermophilic incubator capacity, and our newly-designed anaerobic gassing station is the most efficient anywhere. Plating of thermophilic anaerobes is rather tedious, but we have vessels for culture of 300 plates at a time.

f) Personnel. Dr. Tanner joined the project in June of 1986. He earned his Ph.D. with Dr. R.S. Wolfe at the University of Illinois and then spent four years in industrial research. He has isolated auxotrophs, engineered plasmids, mass cultured methanogens, streamlined laboratory procedures by developing new media and culture apparatus, and worked on the biochemistry of a novel cofactor (mobile factor). Veronica E. Worrell, a graduate student, performed the transformation experiments reported above, isolated mutants and plasmid DNA, and her courses in genetics and biochemistry are strengthening our expertise in the latest techniques. Dr. McCarthy and Doris Kupfer (who joined the project as a research associate in March) have been working on the feasibility of an additive transformation system. Dr. Nagle has been putting our new fermentor systems on line and is characterizing the physiology of some drug-resistant methanogens. There are constant interactions of these workers with other members of the extended research program on anaerobic processes in this department.

### 3. PLANS FOR NEXT YEAR

a) The transformation system will be refined and extended to auxotrophic markers. Mutant selection will continue. Protocols for lysis and preparation of high molecular weight DNA will be optimized.

b) A technique for constructing insertion mutations in M. thermoautotrophicum will be tested. M. thermoautotrophicum chromosomal fragments will be mutagenized in E. coli with the transposon (miniTn10kan). We will attempt to integrate the transposons into the M. thermoautotrophicum chromosome by recombination during transformation with the mutagenized fragments (additive transformation). Potential transposon mutants that have an auxotrophic phenotype will be isolated with our bacitracin enrichment technique. The presence of a transposon in the mutant chromosomes will be demonstrated by Southern blotting using miniTn10kan as a probe. The mutated segments can be recovered by cloning the DNA from the mutants into E. coli and selecting for kanamycin resistance.

c) The pyrimidine analogue-resistant mutants will be utilized to dissect the pyrimidine salvage pathway.

d) A genetically marked M. thermoautotrophicum plasmid will be constructed. Wild type M. thermoautotrophicum DNA will be cloned into the M. thermoautotrophicum plasmid pME2001. We will attempt to isolate plasmids that can complement our collection of auxotrophic mutations.

e) Data will be published in two papers to be submitted in April, and in other publications during the year. We plan to present our work at the Gordon Conference "Molecular Aspects of Methanogenesis" in June, and at the Annual Meeting of the American Society for Microbiology in 1988.

#### 4. DATA SYNOPSIS

##### a) Publications

###### i) in preparation

D.P. Nagle, Jr., R. Teal, and A. Eisenbraun. A 5-fluorouracil-resistant strain of Methanobacterium thermoautotrophicum (Marburg). for submission to J. Bacteriol.

V.E. Worrell, D.P. Nagle, Jr., A. Eisenbraun, and D.R. McCarthy. A natural transformation system for the archaebacterium Methanobacterium thermoautotrophicum. for submission to Nature.

###### ii) related work

R. Teal, and D.P. Nagle, Jr. (1986) Effects of 5-fluorouracil on growth and methanogenesis in Methanobacterium thermoautotrophicum Marburg. Current Microbiology 14, 227-230.

W.J. Jones, D.P. Nagle, Jr., and W.B. Whitman. (1987). Methanogens and the diversity of archaebacteria. Microbiological Reviews 52, 137-158.

##### b) Awards and Honors

M.J. McInerney, University of Oklahoma Associates Distinguished Lecturer, 1986-1987.

##### c) Presentations.

David P. Nagle, Jr. "Genetic engineering and the energy crisis" Presented to the Tinker Society of Professional Engineers and Scientists, Tinker Air Force Base, 26 November, 1986.

Tanner, R.S., and Nagle, D.P., Jr. (1987). Effect of sulfanilamide and folate analogues on the growth of Methanomicrobium mobile. Abstracts of the Annual Meeting of the American Society for Microbiology, Atlanta, GA, 1-6 March, p. 178.

##### d) Graduate Students supported:

i) Veronica E. Worrell (Hispanic female, USA).

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